TOXIN PRODUCTION BY THE FUNGAL PATHOGEN *PYRENOPHORA TRITICI-REPENTIS*, CAUSE OF TAN SPOT DISEASE OF WHEAT

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OF TAN SPOT DISEASE OF WHEAT

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CHAPTER I

INTRODUCTION

The importance of wheat to Oklahoma farmers cannot be overestimated, and the reliance of Oklahoma ranchers on fall-sown wheat as forage for cattle production is a key factor to the agricultural community of the state. As Oklahoma enters the 1990's, two major issues concerning agriculture must be addressed. First, available water, as rainfall or irrigation, may become limiting if the drought which has plagued the Mississippi Valley moves into the Great Plains. Second, the degree to which farmers may rely on pesticides for crop improvement will most likely be reduced over the coming years.

To combat soil erosion and to reduce soil moisture loss, many farmers have turned to reduced, or conservation, tillage practices. Simply stated, the crop residue from the previous planting is left in the field after harvest, and the following crop is planted into the residue after tillage. The advantages of such practices must be weighed against the problems that may occur as a consequence of reduced tillage. Tan spot, a foliar disease of wheat caused by the fungal plant pathogen Pyrenophora tritici-repentis, has become conspicuous in Oklahoma wheat since 1978 as farmers have turned to reduced tillage practices. The fungus is maintained on infected wheat stubble and on other gramineae straw and weeds. After a required cold period to induce sexual reproduction by the fungus, infective propagules are

released into the foliage of wheat planted within the infected residue. When sufficient moisture is present on the leaves, characteristic tan to brown lesions surrounded by a chlorotic halo may develop. As these lesions coalesce and become necrotic, asexual propagules of the fungus may develop and the repetitive disease cycle is initiated. To the degree to which the flag leaf of the wheat plant is infected by P. tritici-repentis, grain yield may be significantly decreased.

As mentioned previously, the degree to which pesticides may be used in the control of plant diseases over the coming decade will most likely decrease as more stringent environmental policy and economic factors dictate agricultural practices. If crop loss to disease is to be controlled, more must be understood about the basic nature of the pathogen. The effects of tan spot on wheat and wheat yields, factors influencing disease progression, and the nutritional and environmental requirements for sexual and asexual reproduction by P. tritici-repentis have been studied extensively. Extensive work monitoring microbial populations on tan spot-infected wheat straw has shown that antagonistic bacterial and fungal species may be identified as biocontrol agents. A basidiomycete, Limonomyces roseipellis, isolated from P. tritici-repentis-infected wheat straw has shown promise as a potential biocontrol organism, and eventually may play a role in decreasing primary inoculum levels of this pathogen. Chemical inhibition of P. tritici-repentis ascocarp development on wheat straw by glyphosate herbicide treatment has been demonstrated.

Ideally, one would incorporate genes for tan spot resistance into agronomically promising lines in the breeding program. Conventional approaches to breeding for resistance to tan spot have been thwarted, however, as the heritability of such resistance is poorly understood.

Reports have indicated that such resistance is conferred both monogenically and polygenically, and further work would be indicated to determine the mode of tan spot resistance inheritance and to identify sources of such resistance which may be exploited.

The means by which P. tritici-repentis isolates cause disease in the field is also controversial. As conidia germinate on susceptible tissue, germ tubes are produced. Infection proceeds as appressoria develop and penetrate the epidermis. A vesicle then develops, from which secondary hyphae arise. These hyphae proceed throughout the leaf mesophyll. It has been proposed that isolates of this pathogen produce a toxin during infection which enhances their ability to infect and become established within susceptible plant tissue. A toxin is defined as a microbially-produced compound which elicits a response in susceptible plants (Durbin, 1981). Toxins are produced by bacterial and fungal plant pathogens, and typical reponses observed are the development of chlorotic and necrotic areas, the disruption of host cellular metabolism; vascular plugging, and most recently, detrimental effects on host nucleic acids have been reported.

Why might one be interested in learning more about toxins produced by plant pathogens? Toxins may be used in screening large numbers of plants for resistance in the place of the pathogen. This screening may be done at the seedling or adult plant stage, at the cellular level, or on the protoplast. The means by which plants are sensitive and / or resistant to a toxin may prove beneficial in identifying potential sources of resistance which may be exploited by the conventional breeding program. Of particular interest is in the utilization of toxins as in vitro selection agents in plant tissue culture, generating novel resistance to plant pathogens which may also be made available to the resistance breeding program

With the goal of further understanding disease mechanisms in the P. tritici-repentis - wheat interaction, the objectives of this study were as follows. First, isolates were screened for the ability to produce toxin(s) in vitro, and methods were developed for the production, isolation, and partial purification of such compounds. Bioassays were outlined to determine the sensitivities of susceptible and resistant wheats, secondary hosts of the pathogen, and other plant species. P. tritici-repentis isolates from Oklahoma wheat and North Dakota wheat, smooth bromegrass, and western wheatgrass were evaluated for their ability to produce toxin in defined liquid culture. Having shown that isolates of this pathogen produce toxin and differ in the ability to do so, toxin production by five P. tritici-repentis isolates over a 21-day period was observed to determine where in the growth cycle (e.g., lag, log, or stationary phase) the toxin was produced. Finally, to identify cultural factors which may play a role in the regulation of toxin production, the effects of glucose concentration, temperature, and light on growth and toxin production were evaluated.

CHAPTER II

LITERATURE REVIEW

Tan spot, a foliar disease of wheat caused by the ascomycetous fungus Pyrenophora tritici-repentis (Died.) Drechs. (syn. P. trichostoma (Fr.) Fckl.), anamorph: Drechslera tritici-repentis (Died.) Shoem. (syn. Helminthosporium tritici-repentis), has been conspicuous in wheat cultivated in Oklahoma since 1978 (Williams, Gough, and Hunger, 1985). Tan spot has also been referred to in the literature as yellow leaf spot, yellow leaf blotch, leaf blotch, wheat leaf blight, and eye spot since its identification on wheat in Japan in 1928 (Hosford, 1981). Also, the causal fungus has been reported under a variety of scientific names for its sexual and asexual states (Table 1).

Tan spot has become a predominant fungal leaf blight in certain wheat producing areas as producers have adopted reduced tillage practices to combat soil erosion and to reduce soil moisture loss (Phillips, Blevins, Thomas, Frye, and Phillips, 1980). The fungus survives on infected wheat stubble and on several other secondary Gramineaceous hosts (Table 2). After a required cold period to induce sexual reproduction, pseudothecia develop, mature, and release infective sexual propagules, or ascospores. When sufficient moisture is present infection occurs on leaves and characteristic tan to brown lesions surrounded by a chlorotic halo develop (Hosford, 1971). Over time, these lesions may coalesce and conidiophores with conidia may form, giving rise to a repetitive infection cycle with

multiple asexual propagules being produced over time (Hosford, 1972). Conidia produced on a standing wheat crop are not considered to be of a magnitude equal to numbers produced on secondary Graminaceous hosts (Hosford, 1971).

The effects of tan spot on wheat have been studied by various means: by a disease-loss relationship derived from single tillers (Rees, Mayer, and Platz, 1981), by comparison of estimates derived from single tillers and plots (Rees, Platz, and Mayer, 1982), and by the comparison of epidemics at different stages of crop development (Rees and Platz, 1983). Various rating systems have been available. Numerical systems expressing percentage of leaf area infected have been used (Hosford, 1971; Hosford and Busch, 1974; Krupinsky, 1982; and Raymond, Bockus, and Norman, 1985). Tan spot has been assessed on a whole plant basis using pictorial keys, such as the Septoria leaf blotch key of James (1971) by various authors (Rees and Platz, 1979; Rees and Platz, 1980; Rees et al., 1982; Rees and Platz, 1983; and Hunger and Brown, unpublished). Both of these techniques are hampered by inherent subjectivity. A more objective method has been used to determine the effect of tan spot on grain yield by examining tiller number and quality, grain yield and quality, and 1000-grain weight (Rees et al., 1981; Rees et al., 1982; and Rees and Platz, 1983).

Among the earliest observed effects of tan spot on Australian wheat is a delay and reduction in tillering. This difference is not maintained to maturity, as only the early delay seems significant (Rees and Platz, 1983). Diseased plants are often smaller, as reported in measurements of peduncle diameter and tiller height. These differences are noticeable only in severe early epidemics and disappear as plants mature. Dry matter in the Australian wheat cultivar 'Olympic' was reduced 36% in comparison to the

control at jointing, but differences at flowering were not significant (Rees and Platz, 1983). Severe tan spot has reduced leaf area at jointing and flowering in the Australian cultivars 'Banks' and 'Olympic'. The leaf area index (LAI) on the susceptible 'Banks' cultivar was reduced 72% at jointing and 36% at flowering in comparison with a non-inoculated control at jointing, but differences were not significant at flowering (Rees and Platz, 1983). A delay in flowering and retarded crop development has been reported. The grain-filling period was reduced by 22% in the cultivar 'Banks' and by 11% in 'Olympic' (Rees and Platz, 1983).

The effect of tan spot on grain yield has been well documented. In the Australian cultivars 'Banks' and 'Olympus', severe tan spot infection reduced yield by 48% and 39% respectively. For 'Banks', it was estimated that late disease resulted in a 35% loss in yield and early infection in a 12.5% loss (Rees and Platz, 1983). Severe yield reductions were reported in Michigan as early as 1952 (Andrews and Klomparens, 1952). Hosford and Busch (1974) reported a loss of 12.9% in grain yield in North Dakota from a combination of P. tritici-repentis and Leptosphaeria avenaria Weber f.sp. triticea T. Johnson. In small plots artifically inoculated with P. tritici-repentis in Montana, losses of 19.7% in 1000-grain weight have been reported (Scharen, Bergman, and Burns, 1976).

Factors Influencing Pathogen Growth and Reproduction

Temperature and Conidiation

One of the most important factors influencing the occurrence and severity of many plant diseases is temperature (Colhoun, 1973). To determine the influence of temperature on the production of asexual

propagules, or conidia, by P. tritici-repentis, Platt, Morrall, and Gruen (1977) observed fifteen isolates of the fungus grown on clarified V8 agar (150ml of V8 juice + 3g $CaCO_3$, clarified by centrifugation, with the volume raised to 1000ml with distilled water and 1.5% agar) or on autoclaved wheat or wheatgrass leaves. On both substrates conidiophores were produced over a range of 10 - 30 C. Mycelial growth was halted at either extreme of 6 and 40 C. On V8 juice medium, conidiation occurred between 14 and 25 C with maximum production at 21 C. The optimal range observed on either autoclaved wheat or wheatgrass tissue was between 19 and 22 C.

Substrate and Conidiation

In the laboratory, conidia are best produced by P. tritici-repentis on V8 juice agar (Platt et al., 1977). Conidia have been produced by the fungus on potato-dextrose agar but not to the extent observed on the V8 juice medium. The concentration of V8 juice in the medium is critical. Fifteen per-cent V8 juice is the optimum for the production of conidia. On 20% V8 juice medium, more aerial hyphae and fewer conidia are produced than on a 15% concentration of V8 juice. On 10% V8 juice medium, both conidiation and mycelial growth were markedly decreased.

Photoperiod and Conidiation

To determine the effect of photoperiod on conidiation by P. tritici-repentis, Platt et al. (1977) examined 25 different photoperiods at 21 C with a light intensity of 4790 lux on 15% V8 juice medium and on autoclaved wheat and wheatgrass tissue. Photoperiods tested ranged from continuous darkness to continuous light in one-hour increments. After 7

days incubation, conidiophores were absent in continuous darkness while only conidiophores were present in continuous light, conidia being absent. Light stimulates conidiophore development when cultures are exposed to as low as 0.5 hour of light per day. The greatest number of conidiophores and conidia developed over a range of 11-13 hours of light followed by a subsequent equal period of darkness.

Light intensity effects both growth and conidiation of P. tritici-repentis. Colony growth and conidiation by six isolates of the pathogen were greatest at 13.3 Wm^{-2} (Platt and Morrall, 1980). The light intensities tested were 219, 135, 44, 29, 13.3, 5.9, 4.4, and 0.3 Wm^{-2}.

Relative Humidity and Conidiation

To determine the effect of relative humidity on conidiation by P. tritici-repentis, Platt and Morrall (1980) examined fungal growth and asexual reproduction under the following conditions. Leaf pieces of wheat and wheatgrass were placed on microscope slides and placed in glass petri dishes lined with filter paper. After autoclaving, the leaf pieces were inoculated at one end with an isolate of the fungus and placed in humidity chambers at various relative humidities (100%, 98%, 97%, 96.5%, 95%, 94%, 93%, 92%, 90%, 85%, and 80.5% relative humidity). Little or no growth was observed at the lower extreme of 80.5% relative humidity. Conidiophores and conidia were formed only at relative humidities of 85% or greater.

The Epidemiology of Tan Spot Disease

The Pathogen Life Cycle

Early work with P. tritici-repentis outlined the intimate association

of this disease with reduced tillage practices in wheat production (Phillips et al., 1980). In nature, the fungus requires an overseasoning on infected wheat straw or stubble for the sexual propagules, or ascospores, to develop within pseudothecia. After a required cold period to induce pseudothecial maturation, infective ascospores are forcibly ejected into the air and onto nearby wheat foliage. If sufficient moisture is present on the leaves, characteristic tan to brown lesions surrounded by a chlorotic halo develop (Hosford, 1971). The length of time that such moisture is present has been associated with resistance to P. tritici-repentis. Highly susceptible wheat has been severely infected after only 6-12 hours of leaf wetness. However, disease severity in resistant wheats increases in varying degrees as moisture duration increases, with most resistant wheats requiring a wet period greater than 48-hour for severe tan spot infection to develop (Hosford, 1982). There appears to be an interaction between the duration of moisture on the leaves and temperature (Hosford, Larez, and Hammond, 1987). Increasing postinoculation wet period and/or temperature has been shown to increase infection and disease development, within limits. After a six hour moisture period at 10 C, infections were few and lesions were limited. Increasing the temperature to 20 and 30 C while moisture duration remained at six hours led to few infections and visible lesions. However, the number and size of lesions increased as the moisture period lengthened (12, 24, and 48 hour) at 10, 20, and 30 C.

As P. tritici-repentis becomes established on the wheat crop, lesions may coalesce and conidiophores with conidia may form, giving rise to the repetitive infection phase (Hosford, 1972). Conidia produced on the standing crop are not considered to be of a magnitude equal to numbers produced on alternative hosts (Hosford, 1971). Following harvest, populations of P.

tritici-repentis have been maintained on wheat straw and stubble. However, other microorganisms are also able to exploit this straw and the developing ascocarps of P. tritici-repentis as nutrient sources (Pfender and Wootke, 1985; 1986; and 1988).

Howard and Morrall (1975) examined the effects of environmental conditions on tan spot progression over time and on airborne spore populations of P. tritici-repentis. In a native prairie in Saskatchewan, Canada, propagules of the fungus were trapped within the confines of a natural ecosystem. When favorable environmental conditions were observed (moisture, temperature, and light), disease intensities were low on all total graminoids in the ecosystem. Both ascospores and conidia were collected over the prairie. The number of conidia recovered was much greater than that of ascospores, although the sexual propagules were detected much earlier than conidia. Also, a marked dirunal periodicity in the trapping of conidia was observed, with peak populations recovered at noon. This periodicity undoubtedly reflects the effect of light and wind on fungus sporulation and spore dispersal (Morrall and Howard, 1975).

Alternative, or Secondary, Hosts of Pyrenophora tritici-repentis

Although most often reported as a disease of wheat, tan spot may become established as a leaf-spotting disease on many alternative, or secondary, hosts of P. tritici-repentis (Hosford, 1971). Over 25 grassy secondary hosts of P. tritici-repentis have been identified (Krupinsky, 1982). Many isolates from these secondary hosts exhibit varying degrees of virulence or aggressiveness, and isolates have been identified which are

more pathogenic on wheat than isolates from diseased wheat (Krupinsky, 1985; 1986; and 1987).

The Influence of Host Resistance on Sexual Reproduction

Results indicate that pseudothecia development may be limited on the straw of resistant wheat. Norman, Bockus, and Raymond (1985) showed that 24.6% fewer pseudothecia developed on resistant 'Red Chief' straw than on susceptible 'Tam-105' in the fall; however, by the following spring, the numbers of pseudothecia on the cultivars were similar.

The *P. tritici-repentis* Infection Process

Infection and Establishment

Until recently, little was known regarding infection by *P. tritici-repentis*. Larez et al. (1986) and Loughman and Deverall (1986) showed that penetration of host epidermal cells occurred by a penetration peg formed from an appressorium. After penetration, vesicle formation occurred and secondary hyphae developed from this vesicle and invaded the mesophyll intercellularly. Various factors, including papillae formation, a common defense mechanism in Gramineae (Sherwood and Vance, 1980), have been implicated in the host resistance response. However, a molecular mechanism influencing resistance to pathogen development appeared more active in resistant than in susceptible wheats (Larez et al., 1986). Resistant and susceptible cultivars of wheat also differed in the extent and speed of growth *P. tritici-repentis* in mesophyll tissue (Loughman and

Deverall, 1986). Three days after inoculation, mean colony radius of the fungus was significantly greater in the susceptible cultivar. This rapid late growth in susceptible tissue may represent a change to a more stimulatory environment induced by the pathogen as it moves through the mesophyll. Continued growth of P. tritici-repentis in susceptible tissue might permit the production of substances toxic to the host so that lesion expansion occurs more rapidly.

Toxin(s) and Tan Spot Disease

Following initial reports regarding the mode of tan spot infection, various groups have proposed that isolates of P. tritici-repentis produce host-specific toxins which may play a role in disease. Cultivar-specific toxicity of culture filtrates of the pathogen was demonstrated by Tomas and Bockus (1987). A molecule of approximately 13,300 molecular weight was purified from culture filtrates and shown to elicit a tanning necrosis after its introduction to susceptible wheat (Tomas, Leach, and Bockus, 1988). Lamari and Bernier (1988; 1989) also identified a 13,300 molecular weight protein which elicits a simlar reponse as the compound identified by Tomas et al. A low-molecular weight (800-1800) toxin, designated Ptr-toxin, was identified by Brown and Hunger (1987a; 1987b; and 1988) which elicits a chlorotic response in detached leaf and toxin injection bioassays. Also, the toxin differentially inhibited the elongation of leaf shoots in a shoot inhibition assay. The relationship of this low-molecular weight toxin with the larger compound isolated by Tomas et al., (1988) and Lamari and Bernier (1988; 1989) is uncertain, and the production of either compound by isolates of P. tritici-repentis during infection has not been demonstrated. However,

Brown and Hunger (1988) demonstrated that isolates differ in their ability to produce toxin in vitro and that Ptr-toxin production may be regulated by environmental factors such as glucose concentration and temperature (Brown and Hunger, unpublished).

Chemical Control of Tan Spot Disease

Fungicide Application for Disease Control

Tan spot development is dependent upon a susceptible host, a virulent form of the pathogen, and favorable environmental factors. Each of these factors must be considered when implementing a disease control strategy. As the disease enters its repetitive conidial phase, fungicide treatment must be repeated to combat infection, and protective fungicides will not prevent development of the fungus or cure infection. Also, fungicide utilization further decreases the narrow profit margin of the wheat crop.

How should control strategies be outlined? As the fungus overwinters on infected crop stubble, clean tillage in a wheat-wheat rotation will prevent early spring infections from ascospores. Under serious conditions, however, rotation to a non-host crop may be indicated (Williams et al., 1985). As mentioned previously, an important part of the disease cycle is that of windblown conidia from alternative (or secondary) hosts and necrotic lesions, so tillage may serve to decrease the rate of inoculum increase but not to prevent it.

The mancozeb fungicides are available for use in the control of tan spot. These are protectant fungicides which act by inhibiting the germination of fungal spores and by killing the developing hyphae. No

therapeutic effect will be observed (Lamey, 1982). In Oklahoma, two fungicide sprays are needed to significantly reduce tan spot disease. Their use, as mentioned, may be cost-limited. Use of broad-spectrum fungicides which also control other wheat leaf blight pathogens may be warranted in the future (Williams et al., 1985).

The objective of fungicide application for tan spot control is to protect the flag leaf, the major contributor to yield. In North Dakota, it is recommended that the first fungicide application be made soon after the flag leaf has emerged if protection is to be complete (Lamey, 1982).

Herbicide Application for the Suppression of Sexual Reproduction

To determine whether a glyphosate-containing herbicide (Roundup, Monsanto Agricultural Company) prepared at labelled rates might inhibit the development of reproductive structures on P. tritici-repentis-infested wheat straw, Sharma et al. (1988) treated infested straw before, or at various times during, ascocarp development. The herbicide completely inhibited ascocarp formation when applied before suitable environmental conditions for their development. Inhibition diminshed as the time delay increased, and was no longer significant if the herbicde was applied to the straw after 10 days of favorable environmental conditions. Interestingly, further experiments conducted show that P. tritici-repentis mycelial growth was not inhibited by the herbicide treatment, indicating a selective activity against the development of sexual fruiting structures.

Biological Control of Tan Spot Disease

Breeding for Disease Resistance

Breeding for resistance to tan spot has proven difficult, and various modes of inheritance of such resistance have been offered. Initial studies undertaken by Nagle, Frohberg, and Hosford (1982) indicated that resistance to P. tritici-repentis did not follow either a monogenic or digenic inheritance pattern, and the authors proposed that resistance was conferred by the presence of multiple genes. Sources of resistance were identified through screening 4,000 wheat selections. Sources included six hexaploids ('BH1146' from Brazil, 'Sundance' from Canada, 'Eklund' from Minnesota, 'Saratovskaja 29' from the Soviet Union, and ND7575 and ND7716 from North Dakota) and five tetraploids (PI166308 from Turkey, PI184526 from Portugal, and 'Wells', D6761, and D6876 from North Dakota). The hexaploid entries ND7575 and ND7716 were advanced breeding lines from the North Dakota State University hard red winter wheat breeding program and lines D6761 and D6876 were from the NDSU durum wheat breeding program.

To detect the presence of one or two resistance genes, the parental, F1, F2, and BC1F1 generations of crosses between a resistance source and a susceptible source were tested in the greenhouse (Nagle et al., 1982). The hexaploid sources were each crossed to the highly susceptible line ND495 and the durums to the cultivar 'Rosette'. Plants were inoculated at the 4-5 leaf stage with a conidial suspension (approximately 2000 conidia / ml). After being placed in a mist chamber for 24 hours, inoculated plants were transferred to a lab bench and rated for disease severity 6-7 days later. Monogenic or digenic inheritance was not indicated based upon the results of

all crosses. Rather than segregating in discrete classes, plants exhibited a continuous distribution of disease severity, indicative of a complex inheritance mode.

Further tests by Nagle et al. (1982) with the hexaploid sources listed above indicated that additive gene effects were most likely of greatest importance in tan spot resistance. Non-additive gene effects, such as dominance plus epistatic effects, were also implicated. Additional sources of tan spot resistance (ND7611, with intermediate resistance, and ND495, with high susceptibility) were included in this study with the hexaploid materials, and tested for resistance inheritance by a modified diallele analysis. Each parent was crossed in one direction only, i.e., with no reciprocal crosses made. The entries were inoculated with a conidial suspension, grown in a randomized compete block design, and evaluated three times at one-week intervals. A disease rating scale was not utilized; instead, the percentage leaf area infected and number of lesions / cm^2 were measured on the fourth leaf. The general combining ability accounted for a significantly greater portion of both traits (% leaf area infected and lesions / unit area) than specific combining ability.

It has also been proposed that a single gene may confer resistance to tan spot. Gough (1982), using the winter wheat cultivar 'Carifen 12' as the female parent (a selection from Chile resistant to tan spot at the adult stage in the field and in the seedling stage in the greenhouse), identified monegenic inheritance of resistance. Crosses were made with the susceptible cultivar 'Tam-101' as the male parent, and residual seed from these crosses representing 141 F3 families, derived from four F1 plants, was evaluated. Plants were inoculated with macerated cultures of P. tritici-repentis isolate PYOK-2. Ten days post-inoculation, plants were

scored as resistant or susceptible and families as homozygous resistant, segregating resistant and susceptible plants, and homozygous susceptible. Only homozygous resistant, which scored much the same as the resistant parent 'Carifen 12' (with dark brown or gray flecks, occasional chlorosis, and limited chlorosis and necrosis at leaf tips) were easily discernible. Families segregating resistant and susceptible and homozygous susceptible were grouped together numerically due to the difficulty in visually separating them. Data combined in this manner was a good fit to a 1:3 ratio, indicating that a single recessive gene conferred tan spot resistance. However, further examination of 30 segregating families proved contradictory. Of this group, 285 plants scored resistant and 148 scored susceptible. The values approximate the expected values for a 3:1 ratio (324.75 : 108.25), indicating that a dominant resistance gene was active.

What is the future of breeding for resistance to tan spot of wheat? Frohberg (1982) outlined the following four areas which breeding programs might investigate and explore: (1) the identification of more sources of resistance, (2) the combination of resistant sources if genetically different, (3) the improvement of inoculation techniques, and (4) the use of waxy leaf genotypes to reduce water retention on the leaf surface. In the greenhouse, the more resistant wheats are not affected by P. tritici-repentis with up to 36 hours of moisture being present on the leaves; susceptible wheats are able to withstand only six hours before tan spotting occurs (Hosford and Busch, 1974).

The Role of Potential Microbial Antagonists in Tan Spot Disease Control

Microorganisms have been isolated and identified which may play a role in the control of tan spot disease by antagonistic activity. Mehdizedegan and Gough (1987) identified compounds produced by *Bacillus lichenformis* and *Pseudomonas fluorescens* which were antagonistic to *P. tritici-repentis*. Such compounds might prove detrimental to *P. tritici-repentis* populations in the phylloplane if they could be applied in an appropriate manner.

As tan spot is intimately associated with diseased wheat residue left in the field, the possibility that microorganisms might interfere with the development of sexual reproductive structures has been investigated. Initially, Pfender and Wootke (1985; 1986) monitored overwintering microbial populations in wheat straw infested with *P. tritici-repentis*. From these populations, a basidiomycete, *Limonomyces roseipellis*, was identified which suppressed ascocarp formation by *P. tritici-repentis*, either by direct antagonism or by competition for nutrients (Pfender, 1987; 1988). To identify other potential biocontrol agents which might play a role in tan spot control, Pfender (1988) has used moisture stress tolerance and aggressiveness, as well as the ability to utilize specific carbon sources such as cellulose and hemicellulose, as indicators of potential of biocontrol capabilities in saprophytic straw-colonizing fungi.

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TABLE I

SCIENTIFIC NAMES BY WHICH *PYRENOPHORA TRITICI-REPENTIS* HAS BEEN REPORTED IN THE LITERATURE (HOSFORD, 1982)

SEXUAL STATE	REPORTED IN
Sphaeria trichostoma Fr.	1823
Pleospora trichostoma (Fr.) Ces. & DeNot.	1861
Pyrenophora trichostoma (Fr.) Fck.	1870
Pleospora tritici-repentis Died.	1903
Pleospora trichostoma f. sp. *tritici-repentis* (Died.) Noack.	1905
Pyrenophora tritici-repentis (Died.) Dresch.	1923
Pyrenophora tritici-vulgaris Dickson	1956
ASEXUAL STATE	
Helminthosporium gramineum Rab. ex. Schlecht. f. sp. *tritici-repentis* Died.	1902
Helminthosporium tritici-repentis (Died.) Died.	1903
Helminthosporium tritici-vulgaris Nisidako	1928
Dreschlera tritici-vulgaris (Nisikado) Ito	1930
Dreschlera tritici-repentis (Died.) Shoem.	1959

TABLE II

ALTERNATIVE, OR SECONDARY, HOSTS OF
PYRENOPHORA TRITICI-REPENTIS
(KRUPINSKY, 1982)

HOST	COMMON NAME
Agropyron cristatum	Crested wheatgrass
Agropyron desertorum	Crested wheatgrass
Agropyron spicatum f. *inerme*	Beardless wheatgrass
Agropyron intermedium	Intermediate wheatgrass
Agropyron spicatum	Bluebunch wheatgrass
Agropyron smithii	Western wheatgrass
Alopecurus arundinaceus	Creeping foxtail
Andropogon gerardii	Big bluestem
Avena sativa	Oats
Bouteloua gracilis	Blue grama
Bromus inermis	Smooth bromegrass
Calamovilfa longifola	Prairie sandreed
Dactylis glomerata	Orchardgrass
Elymus augustus	Altai wildrye
Elymus cinereus	Basin wildrye
Elymus cibericus	Siberian wildrye
Elymus triticoides	Creeping wildrye
Elymus giganteus	Mammoth wildrye
Elymus junceus	Russian wildrye
Hordeum vulgare	Barley
Panicum virgatum	Switchgrass
Phalaris arundinacea	Reed canarygrass
Secale cereale	Rye
Sorghastrum nutans	Indiangrass
Stipa viridula	Green needlegrass

CHAPTER III

PRODUCTION OF A CHLOROSIS-INDUCING TOXIN BY THE FUNGAL PATHOGEN *PYRENOPHORA TRITICI-REPENTIS*, CAUSE OF TAN SPOT DISEASE OF WHEAT

Key Words

Pyrenophora tritici-repentis, *Drechslera tritici-repentis*, host-selective toxins, tan spot, yellow spot, eye spot, foliar diseases of wheat (*Triticum aestivum*).

Abstract

An extraction was developed to isolate toxin from cell-free culture filtrates of *Pyrenophora tritici-repentis*, cause of tan spot disease of wheat. Designated Ptr-toxin, this toxin induces the characteristic chlorosis associated with tan spot disease when inoculated to healthy wheat leaves and differentially inhibits the elongation of wheat seedling coleoptiles. The sensitivity of seven wheat and two non-host (corn and barley) cultivars to Ptr-toxin corresponds with reported field reactions to the pathogen. Seven isolates of *P. tritici-repentis* from wheat (*Triticum aestivum*) in Oklahoma and four from smooth bromegrass (*Bromus inermis*, an alternative host of *P. tritici-repentis*), in North Dakota were evaluated for production of Ptr-toxin *in vitro*.

Results indicate that Ptr-toxin contributes to the expression of

disease symptoms in tan spot of wheat, but is not the sole determinant of P. tritici-repentis pathogenicity. Instead, toxin production may contribute to the virulence (or aggressiveness) of individual isolates of the pathogen. Isolates from the alternative host, smooth bromegrass, appear at least as toxigenic as those from wheat, with the exception of one which appeared more toxigenic than four of the wheat isolates tested.

Introduction

The homothallic ascomycete Pyrenophora tritici-repentis (Died.) Drechs. (syn. P. trichostoma (Fr.) Fckl.), anamorph: Drechslera tritici-repentis (Died.) Shoem. (syn. Helminthosporium tritici-repentis) is the cause of tan spot of wheat (Triticum aestivum L.). Characteristic symptoms of this disease on wheat foliage are brown necrotic lesions surrounded by expansive chlorotic halos. Lesions range from small dark flecks to large tan blotches (Hosford, 1971). This pathogen causes leaf spotting on other cereals and grasses (Krupinsky, 1982) in addition to wheat.

The effects of this disease on wheat and wheat yields, epidemiological factors influencing disease progression, and the nuritional and environmental requirements of the pathogen have been studied extensively in Australia (Rees and Platz, 1979, 1980, and 1983; Rees, Mayer, and Platz, 1981; and Rees, Platz, and Meyer, 1982), Canada (Morrall and Howard, 1975; Platt, Morrall, and Gruen, 1977; and Platt and Morrall, 1980), India (Misra and Singh, 1972), and in the United States (Hosford and Busch, 1974; Hunger and Brown, 1987; Raymond, Bockus, and Norman, 1985). These studies have shown that tan spot severity is related to several factors including the duration of moisture on the leaves, susceptibility or

resistance of the host cultivar, aggressiveness (or virulence) of the fungal isolate, and the growth stage of the host. Reports of the heritability of resistance in the host have been conflicting. Gough (1982) reported that a single recessive gene conferred resistance to P. tritici-repentis in the winter wheat cultivar 'Carifen 12', whereas Nagle et al. (1982) reported that resistance was inherited in a complex fashion in the wheat cultivar 'Edklund'.

Until recently, little was known regarding the infection of P. tritici-repentis or the mode of host resistance to this pathogen. Larez et al. (1986) and Loughman and Deverall (1986) showed that penetration of host epidermal cells occurred by a penetration peg formed from an appressorium. After penetration, vesicle formation occurred and secondary hyphae developed from this vesicle and invaded the mesophyll intercellularly. Various factors, including papillae formation, a common defense mechanism in Gramineae (Sherwood and Vance, 1980), have been implicated in the host resistance response. However, a molecular mechanism influencing resistance to pathogen development appeared more active in resistant than in susceptible wheats (Larez et al., 1986). This resistance may be a response to a toxin produced by P. tritici-repentis as colonization proceeds intercellularly within the mesophyll (Loughman and Deverall, 1986).

The present study was initiated to determine whether a toxin was produced in vitro by P. tritici-repentis and to develop procedures for recovery of toxin from culture filtrates of the pathogen. Methods were developed for the detection and quantification of toxic activity and were used to determine the selective nature of toxin recovered from culture filtrates. To learn whether isolates of the pathogen differed in the ability to produce Ptr-toxin in vitro, 11 isolates of P. tritici-repentis, 7 isolated

from wheat in Oklahoma and 4 from smooth bromegrass (Bromus inermis) in North Dakota, were evaluated for toxigenicity. Abstracts describing parts of this work have been published elsewhere (Brown and Hunger, 1987a, 1987b, and 1988).

Materials and Methods

Production of Culture Filtrates

In a preliminary experiment, three single ascospore isolates of P. tritici-repentis (OKD1, OKD4, and OKD7) were used to study toxin production. In subsequent studies, only OKD4 was used for routine production and isolation of toxin. Five 5mm plugs of the fungus, which was for grown for 5-6 days on 15% clarified V-8 juice agar (150ml of V8 juice + 3g $CaCO_3$, clarified by centrifugation, with the volume raised to 1000ml with distilled water and 1.5% agar), were excised from the edge of a P. tritici-repentis colony. The defined culture medium contained 11.1 mM glucose, 12.5 mM NH_4NO_3, 9.9 mM KNO_3, 7.3 mM KH_2PO_4, 2.0 mM $MgSO_4$, 1.7 mM NaCl, 0.88 mM $CaCl_2$, 3.6 uM $FeCl_3$, 3 uM $ZnSO_4$, 2 uM $MnSO_4$, and 1 uM $CuSO_4$ (Watrud, Hooker, and Koeppe, 1975). One-hundred millilitre aliquots of the defined medium in 250ml flasks were inoculated with the plugs and incubated at 23 C for 11-14 days. The pH of the cultures was 6.3 at inoculation and routinely dropped to 4.0-4.5 over the subsequent two weeks. Filtrate was harvested by filtration through a 0.45 uM filter to remove remaining hyphae.

Toxin Extraction Procedure

Sterile filtrates were concentrated to one-tenth their original volume under vacuum, and two equal volumes of methanol were added to deproteinize the supernatant. The mixture was refrigerated overnight and any precipitate was discarded. Methanol was removed *in vacuo* and the product resuspended in distilled water. This solution was extracted 4-5 times with equal volumes of butanol, combining the organic phases. The aqueous phase was discarded. Under vacuum, this product was taken to dryness, resuspended in distilled water, and tested for toxic activity.

Bioassays

Three bioassays were used to evaluate the toxicity of the end product. Five microlitres of toxin (with distilled water as the control) were placed on a wound made with a sterile metal probe in a detached wheat leaf bioassay (Steiner and Byther, 1971). These leaves were observed after five days for chlorosis. Sensitivity to toxin was tested by a seedling shoot inhibition bioassay, modified from a seedling root inhibition bioassay (Pringle and Braun, 1957). In this bioassay, germinated seeds were placed on Whatman #1 filter paper in dilutions of toxin in sterile nutrient solution or sterile nutrient solution as the control. After 72 hours, shoots were excised and measured, and comparisons made between nutrient solution controls and toxin-amended nutrient solution seedlings. Results were expressed as percentage inhibition of shoot elongation in response to exposure to toxin. Finally, adult plants grown in the greenhouse at growth stage 10.1 on the Feekes scale were used in a toxin injection bioassay (Steiner and Byther, 1971). Two-hundred nanolitres of extracted toxin

(with distilled water as the control) were injected into the culm directly below the apical meristem of the plant. The upper leaves of plants were examined for chlorosis four days after injection.

A combination of these bioassays was used to test for toxin activity. Column chromatography was used to approximate the molecular weight of toxin purified from solvent partitioning and extraction of culture filtrates. BioGels P2 and P4 (BioRad Laboratories, Inc., fractionation ranges of 100-1800 and 800-4000, respectively) were developed with cold (4 C) distilled water at a flow rate of approximately four minutes per six millilitre fraction. Forty fractions from P2 and P4 were collected and tested for toxicity by bioassays. Toxin was autoclaved (1.1 kg/cm^2 at 121 C for 15 minutes) to determine sensitivity to heat and pressure. Toxin was tested at a 1:1, 1:5, and 1:10 dilution in nutrient solution in the seedling shoot inhibition bioassay to determine if differences in toxin sensitivity among host and non-host plants were apparent. The wheat cultivars 'Danne', 'McNair 1003', 'Red Chief', 'Tam-101', 'Tam-105', 'Turkey', and 'Vona' and the barley (Hordeum vulgare) cultivar 'Wintermalt', and corn (Zea mays) cultivar 'Stauffer' were used. The detached leaf bioassay was used to examine the toxin sensitivity of one wheat (cv. 'Tam-101'), four cereals (corn, sorghum, oat, and barley), one legume (peanut), and five vegetables (cantaloupe, cucumber, radish, tomato, and watermelon).

Variation in Toxin Production Between Isolates of P. tritici-repentis

Seven isolates of P. tritici-repentis from Oklahoma (designated OKD1, OKD2, OKD3, OKD4, OKD5, OKD6, and OKD7) representing seven of the eight

ascospores from one ascus (Hunger and Brown, 1987) and four conidial isolates from North Dakota, designated ND4944 SS-1, ND4943 SS-1, ND4939-2, and ND5236-2 SS-2 (Krupinsky, 1987) were used to identify variation in toxigenicity. The seven Oklahoma isolates were originally obtained from wheat and the North Dakota isolates were taken from smooth bromegrass. Variation in pathogenicity had been reported previously for all 11 isolates in separate experiments (Hunger and Brown, 1987; Krupinsky, 1987). The extraction procedure outlined previously was used, with special care taken at each step of the procedure to maintain equal solution volumes between isolates. As an example, 4-100ml aliquots of liquid medium were inoculated for each pathogen isolate. These cultures were incubated at 23C for 14 days. The filtrate was harvested from each culture and the total filtrate pooled. Pooled filtrates were then cold sterilized by passage through a 0.45 um filter. For each isolate, 300ml of cell-free, pooled filtrates were used for the extraction of toxin. At each remaining step in the outlined procedure, the toxin solution was decreased in volume in an equal manner, partitioned with equal volumes of solvent, and resuspended in similar fashion. The final product was taken to dryness under vacuum, resuspended in 60ml distilled H_2O, the flask rinsed twice with 20ml of distilled H_2O, and the rinses added to the 60ml of resuspended Ptr-toxin solution.

Results

Production and Purification of Ptr-toxin

Filtrates from 11-day-old liquid cultures of isolates OKD1, OKD4, and OKD7 were tested by the detached leaf bioassay for toxicity on wheat (cv.

'Tam-101'). Within 72 hours after inoculation, areas of the leaves became chlorotic. This chlorosis was not observed when leaves were inoculated with autoclaved liquid culture medium. The culture filtrate of OKD4 produced the most striking chlorosis on detached leaves in this preliminary study and was chosen for subsequent study of toxin production by P. tritici-repentis.

The product obtained by solvent extraction of OKD4 filtrate was toxic in all three bioassays. Chlorosis was observed in both the detached leaf and toxin injection bioassays, and shoot elongation was inhibited in response to Ptr-toxin as observed in the seedling bioassay. During preliminary extractions, the precipitate obtained with methanol extraction and the aqueous phase left from the butanol partitioning were tested for toxicity. Neither expressed toxicity and were routinely discarded. Solvent-extracted liquid culture medium was tested for toxicity; none was observed.

Physical and Chemical Properties of Ptr-toxin

Ptr-toxin is not sensitive to autoclaving. No significant different was observed between dilutions of autoclaved and non-autoclaved Ptr-toxin in nutrient solution in the seedling shoot inhibition bioassay (data not shown). Refrigeration for one month at 4 C does not have a detrimental effect on Ptr-toxin, as extraction product stored for this period of time exhibited toxicity. Specific fractions collected by column chromatography through BioGels P2 and P4 (after the column bed volumes) exhibited toxicity in the shoot inhibition and detached leaf assays, indicating that the molecular weight of Ptr-toxin lies within the range of 800-1800.

Toxicity and Host-Selectivity of Ptr-toxin

The three bioassays used in this study to determine the selective nature of Ptr-toxin activity were satisfactory. The detached leaf assay is rapid and inexpensive, but requires that healthy tissue be wounded prior to inoculation. This should not create artifact with proper controls. The toxin injection bioassay demonstrates that Ptr-toxin is active on maturing plants, allowing one to disregard concerns about the fragile nature of the seedlings from which shoots were harvested in the previous bioassay. To quantify toxin activity, the seedling shoot inhibition bioassay was most satisfactory. It is not, however, without limitation. Hundreds of seeds are required for each cultivar being tested, eliminating its use in studies of Ptr-toxin activity on precious materials. Special care must be taken to avoid plate contamination and shoot measurement is time-consuming. Work in progress is aimed at modifying or developing a relatively non-destructive assay procedure for continued use in Ptr-toxin studies.

Detached Leaf Bioassay. The reaction of ten plant species including a susceptible and resistant wheat to Ptr-toxin in the detached leaf bioassay is presented (Table 3). The most striking chlorosis was observed in the susceptible wheat cultivar 'Tam-101'. The resistant wheat cultivar 'Red Chief' and the barley cultivar 'Wintermalt' were less sensitive to Ptr-toxin than the susceptible wheat, but the chlorosis observed with all three was dramatic as compared to the other non-host plant species. As stated previously, P. tritici-repentis causes a spotting of numerous Graminaceous species including barley (Krupinsky, 1982), albeit with less severity than on susceptible wheat.

Toxin Injection Bioassay. Adult wheat and barley plants grown in the greenhouse showed varied responses to Ptr-toxin injection. The most chlorosis, a streaking distal to and (to a lesser extent) proximal to the injection site, was observed on the winter wheat cultivar 'Turkey' 48 hours after toxin injection. A moderate, blotchy chlorosis was observed on the winter wheat cultivar 'Tam-101', and limited chlorosis was observed on the winter wheat cultivar 'Red Chief'. No chlorosis was observed in the barley cultivar 'Wintermalt'.

Seedling Shoot Inhibition Bioassay. Significant differences between wheat cultivars, barley, and corn were observed in response to dilutions of Ptr-toxin in the seedling shoot inhibition bioassay (Table 4). As toxin is diluted in sterile nutrient solution, its effect decreases. The degree of sensitivity to Ptr-toxin varies within the plants tested at each dilution. These differences in toxin sensitivity correspond to the reaction of these plants to tan spot disease under field conditions.

Variation in Ptr-toxin Production Between Isolates of P. tritici-repentis

The 11 isolates screened for the production of Ptr-toxin *in vitro* differed in toxigenicity. Significant differences were observed at each dilution and on each wheat tested (Table 5).

Discussion

Previous work has suggested but not demonstrated the involvement of a toxin in tan spot of wheat (Hosford, Larez, and Hammond, 1987; Tomas and

Bockus, 1987). Our work provides substantial evidence for toxin production by P. tritici-repentis. This low-molecular weight (800 < Ptr-toxin < 1800) toxin elicits a chlorotic reaction on both detached seedling and adult wheat leaves, a symptom commonly associated with infection by P. tritici-repentis. However, all symptoms associated with tan spot disease are not reproduced by inoculation with Ptr-toxin, as at no time has necrosis been observed in either plant bioassay. These results are similar to those obtained by Smedegard-Petersen with toxin production by Pyrenophora teres (Smedegard-Petersen, 1977). Toxins A and B produced by this fungal pathogen did not fully reproduce the particular net- and spot-lesions associated with infection, and the author concluded that the toxins did not seem to determine pathogenicity of P. teres isolates but contributed to their virulence. We feel Ptr-toxin production by isolates of P. tritici-repentis influences the severity of tan spot disease but may not be required for the establishment of infection.

As it does not reproduce all symptoms associated with infection on its primary host [as host-specific toxins (Pringle and Scheffer, 1964)], yet does not affect plants not reported as hosts [as non-host-specific toxins (Patil, 1974)], Ptr-toxin activity appears host-selective. Given the broad host range of P. tritici-repentis, it is not surprising that our results show that this toxin is active on its primary host (wheat) and on the alternative host included in this study (barley). Further work aimed at comparing the cytological effect of Ptr-toxin on wheat and other Graminaceous hosts of P. tritici-repentis is suggested.

To determine the significance of any new toxin in plant disease, plant susceptibility to the pathogen and sensitivity to the toxin must be shown. As well, toxin production by numerous isolates of the pathogen must be

demonstrated (Yoder, 1981). Having shown the effect of Ptr-toxin on wheat and barley, we wished to determine whether isolates of P. tritici-repentis differed in toxigenicity and to correlate toxin production with virulence. This work shows that our isolates from wheat and smooth bromegrass differ significantly in the ability to produce Ptr-toxin in vitro. Isolates from the alternative host appear at least as toxigenic as those from wheat in this study. This follows pathogenicity work with the isolates of P. tritici-repentis from wheat and smooth bromegrass used in this study, where isolates from smooth bromegrass were reported to be at least as virulent as those from wheat (Krupinsky, 1987). Subsequent work with P. tritici-repentis from other alternative hosts has shown that isolates of this pathogen from diseased grass leaves (25 different species) caused symptoms on wheat, albeit to varying degrees (Krupinsky, 1988). One isolate from smooth bromegrass, ND4944, appeared to produce more Ptr-toxin than four of the wheat isolates in this study. As well, one of the wheat isolates, OKD5, produced by far the least amount of Ptr-toxin in this report. As the smooth bromegrass isolates did not produce significantly less Ptr-toxin than wheat isolates tested, it would appear that while becoming established on an alternative host, P. tritici-repentis suffers no loss in the ability to produce Ptr-toxin.

Pathogenicity reported in the separate experiments noted does not correspond well with the ability to produce Ptr-toxin in liquid culture. As stated by Yoder (1981), however, toxin production in vitro may be a factor of the artifical substrate; that is, the ability of certain isolates of the pathogen to grow in the defined liquid medium used in this study may have influenced the results obtained. The inherent ability of P. tritici-repentis isolate to produce Ptr-toxin might be masked by the ability of certain

isolates to grow in the medium and under the conditions outlined. Further work *in planta* is indicated before conclusions may be drawn regarding the ability of these isolates to produce toxin during pathogenesis. It should also be noted that other virulence factors associated with *P. tritici-repentis* infection may play significant roles in disease, including the production of cell wall degrading enzymes (e.g. pectinases and cellulases).

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TABLE III

REACTION OF TEN PLANT SPECIES TO PTR-TOXIN, A CHLOROSIS-INDUCING TOXIN PRODUCED BY ISOLATES OF *P. TRITICI-REPENTIS*.

Plant (*Genus species*) and Cultivar	Host [a]	Toxin Reaction [b]
Barley (*Hordeum vulgare*) cv. 'Wintermalt'	Yes	+
Cantaloupe (*Cucumis melo*) cv. 'Habs Best Jumbo'	No	-
Cucumber (*Cucurbita sativus*) cv. 'Poinsett'	No	-
Corn (*Zea mays*) cv. 'Stauffer'	No	-
Peanut (*Arachis hypogea*) cv. 'Pronto'	No	-
Radish (*Raphanus sativa*) cv. 'Cherry Belle'	No	-
Sorghum (*Sorghum bicolor*) cv. 'Sprint'	No	-
Tomato (*Lycopersicon esculentum*) cv. 'Bonne Best'	No	-
Watermelon (*Citrullus vulgaris*) cv. 'Black Diamond'	No	-
Winter Wheat (*Triticum aestivum*) cv. 'Red Chief'	Yes	++
Winter Wheat (*T. aestivum*) cv. 'Tam-101'	Yes	+++

[a] Species reported as a host of *P. tritici-repentis* (Hosford, 1971).

[b] Reaction to Ptr-toxin as determined by the detached leaf bioassay. Symbols: (+++) = severe chlorosis, (++) = moderate chlorosis, (+) = mild chlorosis, and (-) = no chlorosis.

TABLE IV

SENSITIVITY OF WHEAT, BARLEY, AND CORN CULTIVARS TO DILUTIONS OF PTR-TOXIN IN STERILE NUTRIENT SOLUTION

Entry	% Inhibition of shoot elongation [a] Ptr-toxin dilution		
	1:1	1:5	1:10
Corn cv. 'Stauffer'	26 a	17 a	13 ab
Barley cv. 'Wintermalt'	48 b	22 a	5 a
Wheat cv. 'Red Chief'	54 bc	41 bc	28 c
" cv. 'Tam-105'	57 bc	37 b	21 bc
" cv. 'Vona'	60 bc	54 cd	41 de
" cv. 'Danne'	63 c	49 cde	31 cd
" cv. 'McNair 1003'	67 c	52 cde	30 cd
" cv. 'Tam-101'	---	61 e	44 e

[a] Results are expressed as mean percentage inhibition of shoot elongation as compared to nutrient solution controls. Values represent the result of one experiment with three replications and ten subsamples per replication for each entry. The experiment was repeated with similar results. Values followed by common letters are not significantly different according to Duncan's Multiple Range Test (P = 0.05; comparisons may be made only within columns, not between).

TABLE V

VARIATION IN PTR-TOXIN PRODUCTION BETWEEN ELEVEN ISOLATES OF *P. TRITICI-REPENTIS* FROM WHEAT AND SMOOTH BROMEGRASS [a]

	Ptr-toxin dilution							
	1:10				1:25			
	Cultivar tested [b]							
	D	Mc	RC	T	D	Mc	RC	T
Isolate (Rank) [c]								
OKD5 (11)	24 a	37 a	35 a	6 a	12 ab	14 a	16 a	0 a
ND5236 (10)	33 b	57 b	51 b	36 b	0 b	38 b	33 b	7 b
ND4943 (9)	57 c	69 c	61 c	47 c	32 cde	47 c	45 cd	14 b
ND4939 (8)	55 c	70 c	64 c	44 bc	28 bcd	56 de	44 c	14 b
OKD2 (7)	58 c	73 cd	61 c	49 c	15 abc	49 cd	42 c	25 c
OKD4 (6)	66 d	74 cd	72 c	53 c	38 de	61 ef	51 cde	25 c
OKD3 (5)	70 de	77 cd	82 e	68 e	38 de	69 f	56 ef	44 d
ND4944 (4)	79 fg	77 cd	79 e	75 d	47 ef	62 ef	61 f	42 d
OKD1 (3)	75 ef	78 d	84 e	76 de	41 de	64 ef	54 def	46 d
OKD6 (2)	75 ef	79 d	85 e	70 d	48 ef	78 f	58 ef	39 d
OKD7 (1)	84 g	88 e	85 e	85 e	64 f	69 f	63 f	50 d

[a] Results are expressed in percentage inhibition observed in the seedling shoot inhibition bioassay at 1:10 and 1:25 dilutions of Ptr-toxin in sterile nutrient solution. Numbers followed by common letters do not significantly differ at the 0.05 confidence level according to Duncan's Multiple Range Test (comparisons may be made only within cultivars at one dilution, not between).

[b] Ptr-toxin dilutions were tested against the following wheat cultivars: 'Danne' (D), 'McNair 1003' (Mc), 'Red Chief' (RC), and 'Tam-101' (T).

TABLE V (Continued)

[c] Mean rank in toxigenicity, determined from positions in each of the cultivar tests.

CHAPTER IV

TOXIN PRODUCTION BY *PYRENOPHORA TRITICI-REPENTIS*: THE ACCUMULATION OF PTR-TOXIN IN LIQUID CULTURE AND ITS REGULATION BY GLUCOSE CONCENTRATION, LIGHT, AND TEMPERATURE

Key Words

Pyrenophora tritici-repentis, *Drechslera tritici-repentis*, host-specific toxins, tan spot, yellow spot, eye spot, foliar diseases of wheat (*Triticum aestivum*), synthesis and regulation of toxin production.

Abstract

Previous work has demonstrated the production of a low molecular weight toxin (designated Ptr-toxin) by isolates of *Pyrenophora tritici-repentis*, cause of tan spot of wheat (*Triticum aestivum*). Before the mechanisms by which Ptr-toxin is produced are understood, conditions for optimal toxin production must be identified. This study defined glucose concentration, light, and temperature for optimal fungal growth and accumulation of Ptr-toxin in a defined mineral salts-glucose liquid medium. The accumulation of Ptr-toxin in culture was monitored over a 21-day period. The growth of six *P. tritici-repentis* isolates increased significantly over the range of 0.05 to 5.0% glucose. The greatest accumulation of Ptr-toxin was observed at 1 and 2% glucose, and significant differences were found in the specific activity of toxin in the culture

filtrate. The influence of light on growth and Ptr-toxin production were insignificant. Significant increases in fungal dry weights were observed at 23 and 30 C, although the effect of temperature on toxin production remains questionable. The five isolates tested produced variable amounts of Ptr-toxin in culture. The results of our study on the effect of glucose concentration, light, and temperature on Ptr-toxin production reveal much about the specific isolates included in this study but little information upon which generalizations may be based. As such, screening numerous P. tritici-repentis isolates for Ptr-toxin production under one set of conditions appears to be unreliable.

Introduction

Tan spot, a foliar disease of wheat caused by the ascomycetous fungus Pyrenophora tritici-repentis (Died.) Drechs. (syn. P. trichostoma (Fr.) Fckl.), anamorph: Drechslera tritici-repentis (Died.) Shoem. (syn. Helminthosporium tritici-repentis), has been conspicuous in wheat cultivated in Oklahoma since 1978 (Williams, Gough, and Hunger, 1985). Characteristic symptoms of this disease on wheat foliage are brown necrotic lesions surrounded by expansive chlorotic halos. Lesions range from small dark flecks to tan blotches (Hosford, 1971). This pathogen causes leaf spotting on other cereals and grasses (Krupinsky, 1982) in addition to wheat.

Infection by P. tritici-repentis has been investigated by Larez, Hosford, and Freeman (1986) and Loughman and Deverall (1986). Penetration of host epidermal cells occurred by a penetration peg formed from an appressorium. After penetration, vesicles were formed from which

secondary hyphae developed and invaded the mesophyll intercellularly. Various factors, including papillae formation, a common defense mechanism in Gramineae (Sherwood and Vance, 1980), have been implicated in the host resistance response. However, a molecular mechanism influencing resistance to development of P. tritici-repentis appeared more active in resistant than in susceptible wheats (Larez et al., 1986). Resistant and susceptible cultivars of wheat also differed in the extent and growth rate of P. tritici-repentis in mesophyll tissue (Loughman and Deverall, 1986). At three days after inoculation, mean colony radius was significantly greater in the susceptible cultivar. This rapid growth in susceptible tissue may represent a change to a more favorable environment induced by the pathogen as it moves through the mesophyll. Continued growth of P. tritici-repentis in susceptible tissue might permit the production of substances toxic to the host so that lesion expansion occurs more rapidly.

Following initial reports regarding the mode of tan spot infection, various groups have proposed that isolates of P. tritici-repentis produce host-specific toxins which may play a role in disease. Cultivar-specific toxicity of culture filtrates was demonstrated by Tomas and Bockus (1987). A compound of approximately 13,300 molecular weight was purified from culture filtrates and shown to elicit a tanning necrosis after its injection into susceptible wheat (Tomas, Leach, and Bockus, 1988). Lamari and Bernier (1988; 1989) have also identified a protein (MW 13,300) which elicits a similar response as the compound identified by Tomas et al. (1988). We have identified a low-molecular weight (800-1800) toxin, designated Ptr-toxin, (1987; 1987; and 1988) which elicits a chlorotic response in detached leaf and toxin injection bioassays and differentially inhibits the elongation of wheat shoots in a shoot inhibition assay. The

relationship of this low-molecular weight toxin with the larger compound isolated by Tomas et al., (1988) and Lamari and Bernier (1988; 1989) is uncertain, and the production of either compound by isolates of P. tritici-repentis during infection has not been demonstrated. However, we (1988) have shown that isolates of the pathogen wheat and smooth bromegrass differ in their ability to produce Ptr-toxin in vitro, although correlations between toxin production and pathogenicity have been poor.

The present study was initiated to determine the rate at which Ptr-toxin accumulated in defined liquid culture medium over a 21-day period and to compare toxin activities over this time frame. The effects of glucose concentration, light, and temperature on the growth P. tritici-repentis and Ptr-toxin production were investigated to identify optimal conditions for toxin production in vitro. Our objective was to identify conditions that enhance or suppress Ptr-toxin production in defined liquid culture so that the regulation of toxin production by this fungus might be studied.

Materials and Methods

Isolates of P. tritici-repentis

P. tritici-repentis isolates from Oklahoma wheat and North Dakota wheat, smooth bromegrass (Bromus inermis), and western wheatgrass (Agropyron smithii) were used in this study (Table VI). Isolates from Oklahoma wheat were previously shown to exhibit variation in colony color, growth, sporulation, fungicide sensitivity, pathogenicity (Hunger and Brown, 1987) and toxigenicity (Brown and Hunger, 1988). Isolates from North

Dakota were provided by J. M. Krupinsky (Northern Great Plains Research Laboratory, USDA, Mandan, North Dakota).

All pathogen cultures were stored on real potato dextrose agar (Hunger and Brown, 1987) under mineral oil, and fresh isolations were made prior to initiating each experiment. Hyphal tip cultures were established as the isolates grew out from the stored agar slants. Cultures were transferred to clarified V8 juice agar (Raymond, Bockus, and Norman, 1985) and checked for conidia production after five days growth with a subsequent light / dark (12/12 hour) period.

Medium

A defined mineral salts-glucose liquid medium (Watrud, Hooker, and Koeppe, 1975) was used in growth and toxin production experiments. Forty-five (45) ml of liquid medium were placed in 250ml Erlenmeyer flasks, sealed with a foam plug, and autoclaved at 115 C for 10 minutes.

Growth Measurement

Whatman 934AH glass filter paper disks (9cm) were labelled, dried overnight, and pre-weighed. As cultures were inoculated, 3, 5mm disks of fungal hyphae cut with a cork borer from cV8 were placed on each of three filter papers. Papers were autoclaved (121 C, 5 minutes) to remove the agar plugs. Following an overnight period in the drying oven, papers were weighed to determine the dry weight of the inoculum. As each growth experiment demanded, cultures were carefully scraped from the flask, filtered through dried, pre-weighed glass filter papers, and the flask rinsed with 5ml of distilled water to remove additional hyphal fragments. These

papers were dried overnight and weighed to obtain growth measurements in fungal dry weight (mg).

Toxin Extraction Procedure

The filtrates from three replications of each treatment were pooled prior to extraction of toxin. Culture filtrates were harvested by filtration through a 0.45 um filter to remove hyphae remaining after passage through glass filter paper. Two equal volumes of methanol were added to each filtrate to deproteinize the solution. The mixture was refrigerated overnight and any subsequent precipitate was recovered by filtration through Whatman 934AH glass filter paper and discarded. Methanol was removed under vacuum and the product resuspended in 75ml distilled water. This solution was extracted 4 times with 25ml of butanol, combining the organic phases, and the aqueous phase was discarded. This product was taken to dryness under vacuum, resuspended in 75ml distilled water, and tested for toxic activity.

Assay for Ptr-toxin activity

Toxin activity was measured by a seedling shoot inhibition bioassay which was modified from a seedling root inhibition bioassay (Pringle and Braun, 1957). In this bioassay, germinated seeds were placed on Whatman #1 filter paper to which test solutions were added. Test solutions were Ptr-toxin, Ptr-toxin diluted with sterile nutrient solution, and sterile nutrient solution (as the control). Bioassay plates were then sealed and placed in the growth chamber at 23 C with a 16 / 8 photoperiod. After 72 hours, shoots were excised and measured, and comparisons made between

nutrient solution controls and toxin treatments. Results were expressed as percentage inhibition of shoot elongation (activity) in response to exposure to toxin. To compare treatments, specific activity per unit weight (percentage inhibition per mg fungal dry weight) was used.

Effect of Glucose Concentration on Pathogen Growth and Toxin Production

The growth of six isolates of P. tritici-repentis (ND 82-4943 SS-1, ND 88-9813-1 SS-1, ND 88-9819-1 SS-1, ND 88-9954-1 SS-1, ND 88-9886 SS-1, and ND 86-8132 SS-1) was measured in response to 7 concentrations of glucose in the defined liquid medium. The glucose concentrations tested were 0.05, 0.1, 0.5, 1.0, 2.0, 3.0, and 5.0%. These percentages correspond with the following molarities of glucose, respectively: 2.8 mM, 5.5 mM, 27.7 mM, 55.4 mM, 110.1 mM 166.5 mM, and 277.5 mM. Toxin production by two isolates, ND 88-9813-1 SS-1 and ND 86-8132 SS-1, was measured in response to each of the glucose concentrations listed above. Cultures were inoculated with 3, 5mm plugs of fungus taken from a cV8 plate and placed in the growth chamber at 23 C for 13 days (24 hour light, no agitation).

Effect of Temperature on Pathogen Growth and Toxin Production

The growth of six isolates of P. tritici-repentis (ND 82-4943 SS-1, ND 88-9813-1 SS-1, ND 88-9819-1 SS01, ND 88-9954-1 SS-1, ND88-9886 SS-1, and ND 86-8132 SS-1) was measured in response to three temperatures (15, 23, and 30 C) at two glucose concentrations (0.2 and 2.0%; 11.1 mM and 110.1 mM, respectively). Toxin production by two

isolates, ND 88-9813-1 SS-1 and ND 86-8132 SS-1, was measured in response to the temperatures and glucose concentrations listed above. Cultures were inoculated with 3, 5mm plugs of fungus taken from a cV8 plate and placed in the growth chamber for 13 days (24 hour light, no agitation).

Effect of Light on Pathogen Growth and Toxin Production

The growth of six isolates of P. tritici-repentis (ND 82-4943 SS-1, ND 88-9813-1 SS-1, ND 88-9819-1 SS-1, ND 88-9954-1 SS-1, ND 88-9886 SS-1, and ND 86-8132 SS-1) was measured in response to constant light and darkness. Toxin production by two isolates, ND 88-9813-1 SS-1 and ND 86-8132 SS-1, was measured in response to either constant light or dark at three glucose concentrations, 0.02, 0.2, and 2.0% (1.1 mM, 11.1mM, and 110.1 mM, respectively). Cultures were inoculated with 3, 5mm plugs of fungus taken from a cV8 plate and placed in the growth chamber at 23 C for 13 days (no agitation).

Accumulation of Ptr-toxin in Liquid Culture Over Time

Five Oklahoma wheat isolates of P. tritici-repentis (OKD1, OKD3, OKD4, OKD5, and OKD7) were used in this study to measure the accumulation of Ptr-toxin in the defined mineral salts-glucose liquid medium over a 21-day period. Cultures were harvested 3, 6, 9, 13, 17, and 21 days after inoculation with 3, 5mm plugs of the fungus taken from a cV8 plate. Growth and toxin production was measured for each isolate at each sampling date.

Results

Effect of Glucose on Pathogen Growth and Ptr-toxin Production

The growth of P. tritici-repentis isolates increased significantly over the hundred-fold range of glucose concentration tested (Table VII). At each glucose concentration, significant differences were observed in the growth of each isolate tested (analysis not shown). However, these differences were not consistent and varied between glucose concentrations.

The activity of Ptr-toxin produced by P. tritici-repentis at each glucose concentration by isolates ND 88-9813-1 SS-1 and ND 86-8132 SS-1 differed significantly (Table VIII). The higher glucose concentrations, specifically the 1, 2, 3, and 5% concentrations, showed the greatest activity. Specific activities (toxin activity / fungal dry weight [mg]) of Ptr-toxin produced in response to glucose concentration were significantly greatest at 0.1, 1, 2, and 3% glucose. The specific activity of Ptr-toxin produced at 0.1% glucose merits further investigation to determine whether this result is artifact.

Effect of Temperature on Pathogen Growth and Ptr-toxin Production

The isolates tested in this study achieved higher dry weights at 30 C when cultured in 0.2% glucose (Table IX). However, at the higher glucose concentration (2.0%), growth was significantly greater at both 23 and 30 C. The variation in response observed with respect to individual isolates at

each temperature was great; however, specific isolates appear more tolerant of the higher temperature at 30 C. Isolates ND 86-8132 SS-1 and ND 88-9886 SS-1 increased to a greater degree at this higher temperature than the other isolates tested.

No significant differences were observed regarding the specific activity of Ptr-toxin produced by isolates ND 86-8132 SS-1 and ND 88-9813-1 SS-1 at 15, 23, and 30 C (Table X). The greatest amount of Ptr-toxin was produced by ND 86-8132 SS-1 at 23 and 30 C (2% glucose).

Effect of Light on Pathogen Growth and Ptr-toxin Production

Significantly greater growth by P. tritici-repentis was observed at the lowest glucose concentration (data not shown). At 0.02% glucose, these isolates grew significantly greater in the light than in the dark.

No significant difference in the specific activity of Ptr-toxin produced in the light versus the dark was observed in this study (Table X). Although significant differences in the amount of Ptr-toxin produced by the two isolates tested were observed in the light and dark, and at each glucose concentration tested, toxin production does not appear to be induced by light or dark, as differences were likely the result of available glucose in the culture medium.

Accumulation of Ptr-toxin in Liquid Culture Over a 21-Day Period

The variation observed previously in the growth of P. tritici-repentis isolates (Hunger and Brown, 1986) was maintained in this study. Isolates

OKD1, OKD3, and OKD5 grew the most over this period of time, while OKD7, characteristically a slow grower, grew the least.

The specific activities of Ptr-toxin produced by two of the isolates, OKD1 and OKD3, were significantly greatest at days three and six, and day three, respectively (Table XI). No significant differences in the specific activities of Ptr-toxin were observed in this respect for the other three isolates. Ptr-toxin did not accumulate to a significantly greater degree in either isolates OKD5 and OKD7. The greatest accumulation of Ptr-toxin for isolate OKD4 was observed at days 17 and 21, for isolate OKD3 marginally so at days 17 and 21, and for isolate OKD1 at days 13, 17, and 21.

Discussion

Glucose concentration in the defined mineral salts-glucose liquid medium effects both growth and toxin production by isolates of P. tritici-repentis. Growth was greatest at the 5% glucose concentration; however, the specific activity of Ptr-toxin produced by isolates of P. tritici-repentis was significantly decreased at this concentration. Activity and specific activity of Ptr-toxin was greatest at three concentration, 1, 2, and 3% glucose. When comparing these results with those of the growth study, however, the optimal glucose concentration appears to be 2%. While the concentration of glucose in the culture medium may play a role in regulating Ptr-toxin production by any given P. tritici-repentis isolate, generalizations about the nature of such an effect should be avoided, given the variation of the isolates tested in this study.

The ratio of carbon to nitrogen (C:N) in the culture medium has been shown to effect cercosporin production by isolates of Cercospora beticola

(Lynch and Geoghegan, 1979). In this study, only the carbon source (glucose) has been manipulated. In their work, Lynch and Geoghegan (1979) observed an increase in cercosporin production with a maximum C:N ratio of 150. Future studies should identify variation in toxin production in response to carbon and nitrogen manipulation.

Greatest Ptr-toxin accumulation was observed at the higher temperatures in this study (23 and 30 C). A significantly greater amount of Ptr-toxin activity was observed at 30 C (2.0 % glucose) for isolate ND 88-9813-1 SS-1 than at the lower temperatures. ND 86-8132 SS-1 Ptr-toxin activity was greatest at 23 and 30 C (2.0 % glucose). Until further experiments detailing Ptr-toxin production within the range of 23 and 30 C are completed, generalizations regarding the optimal temperature for toxin production may not be made.

Interestingly, the characteristic colony pigmentation of all cultures grown at 30 C changed from green to pink with various amounts of hyaline hyphae present. This increased temperature may influence numerous metabolic activities other than toxin production, and its effect on pathogen growth, development, and metabolism merits further investigation.

Light-grown cultures of Cercospora have been shown to have much higher levels of cercosporin than those grown in the dark, with all other conditions held constant (Balis and Payne, 1971; Fajola, 1978; and Lynch and Geoghegan, 1979). Therefore, light is considered a strong regulator of cercosporin production by isolates of this pathogen. Given the variation observed in our study of light effects on two isolates of the pathogen, a case cannot be made for a regulatory influence of light on Ptr-toxin production by P. tritici-repentis.

Ptr-toxin continues to accumulate in culture over the twenty-one day period tested in this study, with little significant loss in specific activity observed after day 6. If the research goal is to obtain the most Ptr-toxin possible, cultures may be grown for extended period of time (up to three weeks). Culture pH should be carefully monitored over this period of time, and care must be taken to avoid contamination. However, three-day old cultures exhibit sufficient toxin specific activity for assay; therefore, the mechanisms regulating Ptr-toxin production could be studied in a shorter time frame.

Isolates OKD1, OKD3, and OKD4 continued to produce Ptr-toxin over the time period studied, and significant differences were observed in specific activity for isolates OKD1 and OKD3. No significant difference in Ptr-toxin activity or specific activity was observed over the three week period for either OKD5 or OKD7.

As shown in Table VI, work with several of the isolates included in this study is complicated by their instability. OKD5 and ND 86-8132 SS-1 are most variable in culture, and sector readily. This problem is made more difficult as sectors are not always visually identifiable. Inoculum taken from sectoring colonies may influence experimental results, specifically by increasing variation. Attempts to maintain sub-cultures from colony sectors have proved difficult, and the majority no longer grow after two to three transfers. However, a small collection of such spontaneous variants is available for future study.

The results of our study on the effect of glucose concentration, light, and temperature on Ptr-toxin production reveal much about the specific isolates included in this study but little information upon which generalizations may be based. The influence of each of these parameters

differed greatly among the isolates. Similar difficulties have been encountered in work with cersporin production by Cercospora species and isolates (Jenns, Daub, and Upchurch, 1989). We have identified conditions which enhance the production of Ptr-toxin in vitro, but conclusions about the regulation of such production by these conditions would be premature. Screening numerous isolates of P. tritici-repentis for Ptr-toxin production under one set of conditions is unreliable, and as shown by our data, the reliability of correlating in vitro toxin production with isolate pathogenicity may be complicated by the variation observed in response to cultural parameters. However, specific isolates have been identified which may be exploited for detailed study of toxin regulation, which may lead to an understanding of the molecular basis for Ptr-toxin production by P. tritici-repentis.

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TABLE VI

PHENOTYPE OF P. TRITICI-REPENTIS ISOLATES USED IN GROWTH AND PTR-TOXIN PRODUCTION STUDIES

Isolate	Host[a]	Origin	Conidia[b]	Pigmentation	Stability[c]
OKD1	Wheat	Ascospore	Yes	Dark green	+++
OKD3	Wheat	Ascospore	No[d]	Light orange	++
OKD4	Wheat	Ascospore	No	Bright orange	+++
OKD5	Wheat	Ascospore	Yes	Dark green	+
OKD7	Wheat	Ascospore	No	None	++
ND 82-4943 SS-1					
	Smooth bromegrass				
		Conidial	Yes	Dark green	+++
ND 88-9813-1 SS-1					
	Wheat	Conidial	Yes	Dark green	++
ND 88-9819-1 SS-1					
	Wheat	Conidial	Yes	Dark green	+++
ND 88-9954-1 SS-1					
	Wheat	Conidial	Yes	Dark green	++
ND 88-9886 SS-1					
	Wheat	Conidial	Yes	Dark green	+++
ND 86-8132 SS-1					
	Western wheatgrass				
		Conidial	Yes	Dark green	+

[a] Host plant from which isolation was made. North Dakota isolates provided by J.M. Krupinsky (Northern Great Plains Research Laboratory, USDA, Mandan, North Dakota).

[b] Isolate demonstrates ability to produce conidia under conditions outlined by Ramond, Bockus, and Norman (1985).

[c] Subjective rating regarding the degree and frequency of cultural variability (i.e., sectoring) observed with particular isolates. Scale: +++ = isolate very stable in culture, rarely sectors, ++ = isolate stable in culture, sectors occasionally, and + = isolate unstable in culture, sectors frequently.

TABLE VI (Continued)

[d] OKD3 produces conidia at a reduced rate as compared to other conidia-producing isolates included in this study (Hunger and Brown, 1986).

TABLE VII

EFFECT OF GLUCOSE CONCENTRATION ON
P. TRITICI-REPENTIS GROWTH [a]

Glucose Concentration (%)	Isolate 4943	8132	9813	9819	9886	9954
0.05	14.2	13.2	13.0	14.5	13.4	12.3
0.10	21.2	21.5	15.0	20.9	17.2	16.0
0.50	36.1	38.4	17.2	30.4	26.2	26.2
1.00	45.9	45.6	27.6	39.3	33.0	31.5
2.00	73.1	64.4	49.2	60.4	68.2	61.3
3.00	101.0	98.8	63.0	73.8	72.4	76.6
5.00	131.6	126.9	135.7	95.5	91.4	119.6
Slope	23.6	22.5	23.7	16.1	16.3	22.0
y-Intercept	21.1	21.0	6.4	21.0	18.9	13.7

[a] Mean dry weight (mg) of fungus harvested 13 days post-inoculation.

TABLE VIII

EFFECT OF GLUCOSE CONCENTRATION ON THE ACCUMULATION AND ACTIVITY OF PTR-TOXIN IN DEFINED LIQUID MEDIUM

Treatment	Activity[a]	Dry weight[b]	Specific Activity[c]
0.05% glucose	0 A	39.3	0.000 a
0.1 % glucose	7 AB	54.8	0.128 abc
0.5 % glucose	9 AB	83.4	0.108 ab
1.0 % glucose	20 ABC	109.3	0.183 bc
2.0 % glucose	42 C	170.5	0.246 c
3.0 % glucose	32 BC	242.8	0.132 abc
5.0 % glucose	36 C	393.9	0.091 ab

[a] Mean percent inhibition of shoot elongation by toxin produced by isolates ND 88-9813-1 SS-1 and ND 86-8132 SS-1. Numbers followed by common capital letters do not differ significantly according to Duncan's Multiple Range Test (P = 0.05).

[b] Mean dry weight of fungus harvested from each treatment for each isolate.

[c] Specific activities calculated as described previously. Numbers followed by common lower case letters do not differ significantly according to Duncan's Multiple Range Test (P = 0.05).

TABLE IX

EFFECT OF TEMPERATURE AND GLUCOSE CONCENTRATION ON PTR-TOXIN PRODUCTION AND ACTIVITY BY P. TRITICI-REPENTIS

Isolate	Treatment	Activity[a]	Weight[b]	Specific Activity[c]
8132	0.2 % glucose, 15 C	5 A	58.2	0.086 a
	2.0 % glucose, 15 C	9 A	126.7	0.071 a
	0.2 % glucose, 23 C	9 A	53.3	0.169 a
	2.0 % glucose, 23 C	35 CD	193.5	0.181 a
	0.2 % glucose, 30 C	24 BC	99.8	0.240 a
	2.0 % glucose, 30 C	46 D	208.3	0.221 a
9813	0.2 % glucose, 15 C	5 A	24.9	0.201 a
	2.0 % glucose, 15 C	10 A	83.2	0.120 a
	0.2 % glucose, 23 C	2 A	35.7	0.056 a
	2.0 % glucose, 23 C	7 A	98.2	0.071 a
	0.2 % glucose, 30 C	6 A	59.1	0.102 a
	2.0 % glucose, 30 C	14 AB	150.0	0.093 a

[a] Mean percent inhibition of shoot elongation by toxin produced by isolates ND 88-9813-1 SS-1 and ND 86-8132 SS-1. Numbers followed by common capital letters do not differ significantly according to Duncan's Multiple Range Test (P = 0.05).

[b] Mean dry weight of fungus harvested for each isolate at each treatment.

[c] Specific activity calculated as described previously. Numbers followed by common lower case letters do not differ significantly according to Duncan's Multiple Range Test (P = 0.05).

TABLE X

EFFECT OF LIGHT AND GLUCOSE CONCENTRATION ON PTR-TOXIN PRODUCTION AND ACTIVITY BY P. TRITICI-REPENTIS

Isolate	Treatment	Activity[a]	Weight[b]	Specific Activity[c]
8132	0.02 % glucose, dark	4 AB	15.6	0.256 a
	0.20 % glucose, dark	12 BC	95.4	0.126 a
	2.00 % glucose, dark	25 DE	164.9	0.152 a
	0.02 % glucose, light	3 AB	18.9	0.159 a
	0.20 % glucose, light	5 AB	80.5	0.062 a
	2.00 % glucose, light	19 CD	220.1	0.086 a
9813	0.02 % glucose, dark	4 AB	20.4	0.196 a
	0.20 % glucose, dark	5 AB	42.6	0.117 a
	2.00 % glucose, dark	45 F	147.2	0.306 a
	0.02 % glucose, light	0 A	21.0	0.000 a
	0.20 % glucose, light	3 AB	72.6	0.041 a
	2.00 % glucose, light	35 EF	139.7	0.251 a

[a] Mean percent inhibition of shoot elongation by toxin produced by isolates ND 88-9813-1 SS-1 and ND 86-8132 SS-1. Numbers followed by common capital letters do not differ significantly according to Duncan's Multiple Range Test (P = 0.05).

[b] Mean dry weight of fungus harvested for each isolate at each treatment.

[c] Specific activity calculated as described previously. Numbers followed by common lower case letters do not differ significantly according to Duncan's Multiple Range Test (P = 0.05).

TABLE XI

ACCUMULATION OF PTR-TOXIN IN CULTURE BY FIVE *P. TRITICI-REPENTIS* ISOLATES OVER A TWENTY-ONE DAY PERIOD

Isolate	Day	Activity[a]	Weight[b]	Specific Activity[c]
OKD1	3	28 A	43.9	0.638 a
	6	32 AB	57.3	0.558 a
	9	29 A	86.1	0.337 b
	13	35 ABC	123.1	0.284 b
	17	44 BC	141.2	0.312 b
	21	46 C	226.7	0.203 b
OKD3	3	35 A	53.2	0.658 a
	6	33 A	86.9	0.380 b
	9	39 A	111.3	0.350 b
	13	38 A	126.8	0.296 b
	17	54 B	132.2	0.405 b
	21	44 AB	278.0	0.158 c
OKD4	3	32 AB	58.6	0.538 a
	6	16 A	60.0	0.266 a
	9	28 AB	88.5	0.316 a
	13	29 AB	106.2	0.273 a
	17	47 BC	163.7	0.287 a
	21	54 C	168.8	0.320 a
OKD5	3	25 A	48.5	0.515 a
	6	18 A	74.0	0.243 a
	9	40 A	90.8	0.441 a
	13	38 A	135.5	0.280 a
	17	35 A	160.8	0.218 a
	21	46 A	233.4	0.197 a

TABLE XI (Continued)

OKD7	3	14 A	47.1	0.297 a
	6	17 A	47.0	0.362 a
	9	11 A	54.9	0.200 a
	13	19 A	56.3	0.337 a
	17	22 A	56.8	0.387 a
	21	26 A	62.0	0.419 a

a Mean percent inhibition of shoot elongation by toxin produced by the five isolates. Numbers followed by common capital letters do not differ significantly according to Duncan's Multiple Range Test (P = 0.05; comparisons may only be made within specific isolates at sampling dates.

b Total dry weight of fungus harvested at each sampling date.

c Specific activity calculated as described previously. Numbers followed by common lower case letters do not differ significantly according to Duncan's Multiple Range Test (P = 0.05).

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APPENDICES

APPENDIX A

MEDIA PREPARATION

Clarified V8 juice agar (15% cV8).

3 g $CaCO_3$
150 ml clarified V8 juice
15 g agar

Combine V8 juice and $CaCO_3$. Clarify mixture by centrifugation at 3000 rpm for five minutes. Heat 150 ml of clarified V8 juice and 800ml of distilled water to boiling. Add 15 g agar to mixture with stirring. Reduce to low heat with stirring until agar is dissolved. Carefully bring volume to one liter and prepare medium for autoclaving.

Hoagland's nutrient solution.

The following stock solutions may be prepared in advance, autoclaved, and stored at 4 C until used:

Stock solution A: 27.41 g KNO_3
7.38 g KH_2PO_4
54.63 g $MgSO_4 * 7H_2O$
1.75 g NaCl
Total volume of stock A in distilled water = 250 ml.

Stock solution B: 113.83 g $Ca(NO_3)_2 * 3H_2O$
Total volume of stock B in distilled water = 250 ml.

Stock solution C: 1.41 g H_3BO_3
0.02 g $CuCl_2 * 2H_2O$
0.15 g $ZnCl_2$
2.50 g $FeCl_3 * 6H_2O$
Total volume of stock C in distilled water = 500 ml.

To prepare one litre of Hoagland's nutrient solution, combine 5 ml of Stock solution A, 4 ml of Stock solution B, and 0.1 ml of Stock solution C in 1000 ml of distilled water solution. Autoclave prior to use or storage.

Real potato dextrose agar (rPDA:

200 g potatoes, peeled and sliced.
20 g dextrose
15 g agar

Cook potatoes in 500 ml of distilled water for 45 minutes. Cool and decant through several layers of cheesecloth to obtain potato broth. Increase volume of potato broth to 900 ml, add dextrose with stirring, and bring to a boil. Add agar with constant stirring and bring to a low boil. Remove from heat, raise volume to 1000 ml with distilled water, and

Mineral salts-glucose liquid defined medium (WHK):

The following stock solutions may be prepared and autoclaved prior to medium preparation:

WHK Stock solution A: 1.29 g $CaCl_2 * 2H_2O$
10.00 g NH_4NO_3
10.00 g KNO_3
9.93 g KH_2PO_4
4.92 g $MgSO_4 * 7H_2O$
0.99 g NaCl
Total volume of WHK stock A in distilled water = 1 l.

WHK Stock solution B: 0.97 g $FeCl_3 * 6H_2O$
Total volume of WHK stock B in distilled water = 1 l.

WHK Stock solution C: 0.86 g $ZnSO_4 * 7H_2O$
Total volume of WHK stock C in distilled water = 1 l.

WHK Stock solution D: 0.30 g $MnSO_4$
Total volume of WHK stock D in distilled water = 1 l.

WHK Stock solution E: 0.25 g $CuSO_4 * 5H_2O$
Total volume of WHK stock E in distilled water = 1 l.

To prepare one liter of mineral salts-glucose liquid medium, combine the following in 1000 ml of distilled water solution: 100 ml WHK stock A, 1 ml WHK stock B, 1 ml WHK stock C, 1 ml WHK stock D, 1 mk WHK stock E, and 20 g glucose. Mix well to dissolve glucose and pour into flasks at volumes desired. Autoclave for 10 minutes at 115 psi.

APPENDIX B

BIOASSAYS: PREPARATION AND PROCEDURES

Laboratory assays:

Detached leaf bioassay. Leaves of seedling plants were detached, cut into 4 cm lengths, and placed between moistened paper towels. To set up this bioassay, the tip and basal portions of the leaves were fixed to a glass slide with adhesive tape, trimmed as necessary, and the direction of transpiration noted on the slide. A wound was made distal to the tip end of the leaf with a metal probe and the slide was placed in a petri dish lined with three sheets of Whatman #1 filter paper generously moistened with distilled water. Leaves were then inoculated with 5 ul of toxin at the wound site. Controls were inoculated with 5 ul of distilled water. The dishes were carefully sealed and placed in the growth chamber at 23 C with constant light. Symptom development (e.g., toxic activity) was assessed as chlorosis developed on the leaves. Quite often, this chlorotic activity was observed as either a streaking, spotting, or mottling as compared to the distilled water controls (Steiner and Byther, 1972; 1976).

Seedling shoot inhibition bioassay. To successfully use this bioassay, the first step involves the surface disinfestation of the seed which is to be used. To do so, seed was washed with constant agitation in a 10 % chlorox solution, with Tween-20 added as a surfactant. After one hour, seed was carefully rinsed with several changes of distilled water. Following this, seed was washed for one hour in cold (4 C) distilled water with constant agitation. After draining off excess water, seed was allowed to germinate for 72 hours between several sheets of moistened cheesecloth.

To prepare the bioassay, petri plates were lined with three sheets of Whatman #1 filter paper, to which various test solutions were added. Solutions included toxin, dilutions of toxin in sterile nutrient solution, distilled water, and sterile nutrient solution. Ten seeds were placed on

each plate, taking care to select seeds with a primary shoot of approximately 1 - 3 mm in length. These plates were sealed and placed in the growth chamber at 23 C with a 16 / 8 photoperiod. Toxin activity was determined by measuring the shoots and calculating percentage inhibition of shoot elongation, comparing test and control plates (Comstock and Scheffer, 1972; Kuo, Yoder, and Scheffer, 1970; Pringle and Brauer, 1957; and Scheffer and Pringle, 1964).

Even with the stringent surface disinfestation, the occasional seed has become contaminated during this assay. These seeds were not included in shoot measurements. A careful inspection of the seed to be used prior to beginning this assay may identify potential problems in contamination.

Greenhouse bioassay:

Toxin injection bioassay. Healthy 28-day-old plants were used. Toxin solution (0.2 ml) was injected into the plant directly below the leaf whorl, with distilled water used as the control. Plants were examined daily for symptom expression (i.e., chlorotic spotting, streaking, or mottling) with ratings determined 48 and 96 hours after inoculation (Steiner and Byther, 1971).

VITA[2]

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Doctor of Philosophy

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